U.S. PATENT APPLICATION

FOR

Anti-Cancer Anthracycline Drug-Antibody Conjugates

Inventor: G.L. Griffiths

Anti-Cancer Anthracycline Drug-Antibody Conjugates

FIELD OF THE INVENTION

[0001] The invention relates to therapeutic conjugates with the ability to target various antigens. The conjugates contain a targeting moiety and a chemotherapeutic drug. The targeting and the chemotherapeutic drug are linked via a linker comprising an intracellularly cleavable moiety.

BACKGROUND OF THE INVENTION

[0002] For many years it has been a goal of scientists in the field of specifically targeted drug therapy that antibodies could be used for the specific delivery of chemotherapy drugs to human cancers. Realization of such a goal could finally bring to cancer chemotherapy the concept of the magic bullet. A significant advance toward achieving this goal came with the advent of the hybridoma technique of Köhler and Milstein in 1975, and the subsequent ability to generate monoclonal antibodies (mAbs). During the past 25 years mAbs have been raised against many antigenic targets that are over-expressed on cancerous cells. Either alone, or as conjugates of drugs, toxins, radionuclides or other therapy agents, many mAbs have been tested pre-clinically, and later in clinical trials. Generally, mAbs by themselves, often termed "naked mAbs," have not been successful at making long-term survivorship the norm in patients with solid tumors, although survival advantages have lately been seen with mAb treatments directed against both breast and colon cancer (mAbs against HER2-*neu* and 17-1A, respectively). With hematological malignancies more success is being achieved with naked mAbs, notably against the B-cell lymphomas (mAbs against CD20 and CD22 on the surface of B-cells).

[0003] It appears self-evident, however, that the use of conjugates of tumor-associated mAbs and suitable toxic agents will be more efficacious than naked mAbs against most clinical cases of cancer. Here, a mAb also carries a toxic agent specifically to the diseased tissue, in addition to any toxicity it might inherently have by virtue of natural or re-engineered effector functions provided by the Fc portion of the mAb, such as complement fixation and ADCC (antibody dependent cell cytotoxicity), which set mechanisms into action that may result in cell lysis. However, it is possible that the Fc portion is not required for therapeutic function, as in the case of mAb fragments, other mechanisms, such as apoptosis, inhibiting angiogenesis, inhibiting metastatic activity, and/or affecting tumor cell adhesion,

may come into play. The toxic agent is most commonly a chemotherapy drug, a particle-emitting radionuclide, or a bacterial or plant toxin. Each type of conjugate has its own particular advantages. Penetrating radionuclides and the bacterial and plant toxins are extremely toxic, usually orders of magnitude more toxic than standard chemotherapy drugs. This makes the former two useful with mAbs, since in a clinical situation the uptake of mAbs into diseased tissue is extremely low. The low mAb tumor uptake in clinical practice and the relatively low toxicity profile of cancer chemotherapy drugs, combined, is a major reason why mAb-drug conjugates have failed to live up to their promise, to date.

[0004] In preclinical animal xenograft models, set up to study human cancer, many mAb conjugates have been described which are able to completely regress or even cure animals of their tumors. However, tumor uptakes of mAb conjugates in many of these animal xenograft models are often in the 10-50% injected dose per gram of tissue range, whereas in the clinical situation, tumor uptakes in the 0.1-0.0001 % injected dose per gram of tissue are more normal. It is no surprise, then, that mAb conjugates made with the more toxic radionuclides and toxins have generally fared somewhat better, clinically, than the corresponding mAb-drug conjugates with standard chemotherapeutic drugs. However, radionuclide mAb conjugates can often produce great toxicity due to the presence of a great excess of circulating, decaying radioactivity compared to tumor-localized activity. Toxin-mAb conjugates have suffered from the dual drawbacks of great non-target tissue damage and great immunoreactivity toward the plant or bacterial protein that is generally used. Whereas mAbs can now be made in human or in humanized (complementarity-determining region-grafted) forms, de-immunization of the toxin part of any conjugate will likely remain a significant obstacle to progress.

[0005] Despite the lack of necessary efficacy in a clinical setting seen to date, mAb-drug conjugates still have compelling theoretical advantages. The drug itself is structurally well defined, not present in isoforms, and can be linked to the mAb protein using very well defined conjugation chemistries, often at specific sites remote from the mAbs' antigen binding regions. MAb-drug conjugates can be made more reproducibly than chemical conjugates involving mAbs and toxins, and, as such, are more amenable to commercial development and regulatory approval. For such reasons, interest in drug conjugates of mAbs has continued despite the disappointments encountered. In some recent instances, however, preclinical results have been quite promising. With continuing refinements in conjugation chemistries, and the ability to remove or reduce immunogenic properties of the mAb, the

elusive promise of useful mAb-drug conjugates for clinical cancer therapy are being newly considered.

[0006] Relevant early work on mAb-drug conjugates found during *in vitro* and *in vivo* preclinical testing that the chemical linkages used often resulted in the loss of a drug's potency. Thus, it was realized many years ago that a drug would ideally need to be released in its original form, once internalized by a target cell by the mAb component, in order to be a useful therapeutic. Work during the 1980s and early 1990s then focused largely on the nature of the chemical linker between the drug and the mAb. Notably, conjugates prepared using mild acid-cleavable linkers were developed, based on the observation that pH inside tumors was often lower than normal physiological pH (U.S. Patent Nos. 4,542,225; 4,569,789; 4,618,492; and 4,952,394). This approach culminated in a landmark paper by Trail *et al.* (*Science 261*:212-215 (1993)) showing that mAb-doxorubicin (DOX) conjugates, prepared with appropriate linkers, could be used to cure mice bearing a variety of human tumor xenografts, in preclinical studies. This promising result was achieved with an antibody (termed BR96) that had a very large number of receptors on the tumor cells being targeted, the mAb-drug conjugate was highly substituted (6-8 DOX residues per unit of mAb), and the conjugate was given in massive doses on a repeat basis.

[0007] In the clinical situation, tumor uptakes of mAbs would be much lower, and since this variable was something that had to be addressed, more toxic drugs, would be needed to achieve a desirable therapeutic effect. More toxic drugs were used in the development of several distinct mAb-drug conjugates (U.S. Patent Nos. 5,208,020; 5,416,064; 5,877,296; and 6,015,562). These efforts use drugs, such as derivatives of maytansinoids and calicheamicin, which are essentially too toxic to be used in standard chemotherapy. Conjugation to a mAb enables relatively more of the drug to be targeted to a tumor in relation to the often non-specific cell and protein binding seen with chemotherapy alone. The exquisite toxicity of drugs such as these might overcome the low levels of tumor-targeted mAb seen clinically, due to the low level of antigen binding sites generally seen on tumor targets. In preclinical studies, cures of mice bearing human tumor xenografts were seen at much lower doses of mAb-drug conjugate, than seen previously with mAb-drug conjugates using standard drugs, such as DOX (Liu *et al., Proc. Natl. Acad. Sci. USA 93*:8616-8623 (1996) and Hinman *et al., Cancer Res. 53*:3336-3342 (1993)). In the case of the maytansinoid-mAb conjugates (Liu), the amount of conjugate needed for therapy was over > 50-fold less than needed previously with DOX conjugates (Trail, *supra*).

[0008] During development of these conjugates the linker between drug and mAb was thought to be critical for retention of good anti-tumor activity both *in vitro* and *in vivo*. The cited conjugates were made with an intracellularly-cleavable moiety (hydrazone) and a reductively labile (disulfide) bond between the drug and the mAb. While the hydrazone bond is apparently stable to *in vivo* serum conditions, normal disulfide bonds were found to be not stable enough for practical use. Conjugates were made that replaced a standard disulfide linkage with a hindered (geminal dimethyl) disulfide linkage in the case of the calicheamicins, or a methyl disulfide in the case of the maytansinoids. While this work was being done, separate work also continued on newer anthracycline-substituted mAb conjugates. In the case of newer DOX conjugated mAbs, it was found that superior results could be obtained by incorporating just a hydrazone as a cleavable unit, and attaching DOX to mAb via a thioether group, instead of a disulfide (U.S. Patent No. 5,708,146). When linked in such a manner, and also using a branched linker capable of doubling the number of DOX units per MAb substitution site, an approximate order of magnitude increase in the efficacy of the new DOX-MAb conjugates were obtained (King *et al.*, *Bioconjugate Chem. 10*:279-288, (1999)).

SUMMARY OF THE INVENTION

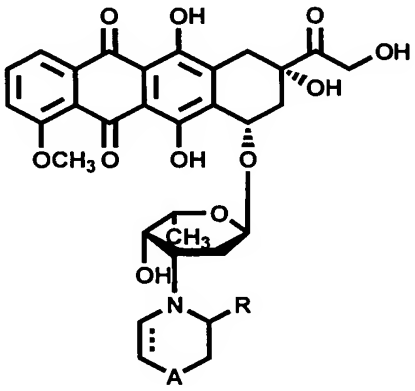
[0009] The present invention is directed to new internalizing antibody conjugates of anthracycline drugs. Specific embodiments are exemplified by conjugates of doxorubicin (DOX), epirubicin, morpholinodoxorubicin (morpholino-DOX), cyanomorpholino-doxorubicin (cyanomorpholino-DOX), and 2-pyrrolino-doxorubicin (2-PDOX). 2-PDOX is particularly toxic, incorporating an enamine in its structure, which can act not only as an intercalator and topoisomerase inhibitor, but also as an alkylating agent having increased toxicity. Like DOX, 2-PDOX has relatively good aqueous solubility which means that it can be coupled to mAbs in multiply substituted amounts without precipitation of the mAb. The drugs described in detail below are consistently substituted at an average of 8 (typically measured at 7 – 9) drug moieties per molecule of mAb. The number of drugs, however, may also range between 6 to 10 molecules per molecule of mAb.

[0010] In one aspect, the invention relates to an immunoconjugate comprising a targeting moiety, an anthracycline drug and a linker binding the targeting moiety via a thiol group and the anthracycline chemotherapeutic drug via an intracellularly-cleavable moiety.

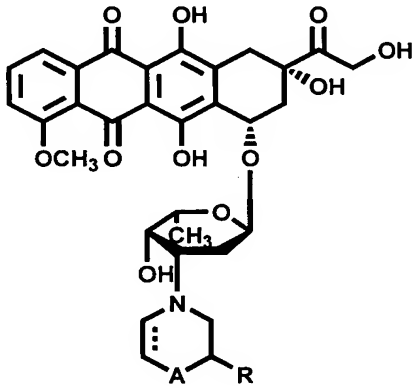
[0011] In a preferred embodiment of the present invention, the targeting moiety is a mAb, the anthracycline chemotherapeutic drug is DOX, 2-PDOX, morpholino-DOX and morpholinocyano-DOX, and the intracellularly-cleavable moiety is a hydrazone.

[0012] In another aspect, the invention relates to an immunoconjugate comprising a disease-targeting antibody and an anthracycline chemotherapeutic drug. Many hundreds of examples of anthracycline drugs have been synthesized over the last 30-40 years or so, and they are discussed in detail elsewhere (*see: Anthracycline Antibiotics; New Analogs, methods of Delivery, and Mechanisms of Action, Waldemar Priebe, Editor, ACS Symposium Series 574, American Chemical Society, Washington DC, 1994*). Such analogs are envisaged as within the scope of the current invention.

[0013] In a preferred embodiment, the invention relates to an immunoconjugate comprising a disease-targeting antibody and an anthracycline chemotherapeutic drug of the formulae I and II:



(I)



(II)

wherein, A is nothing or it may be selected from the group consisting of NH, N-alkyl, N-cycloalkyl, O, S, and CH_2; the dotted line denotes a single or a double bond; and R is H or CN; and a linker binding the targeting moiety via a sulfide group and the anthracycline chemotherapeutic drug via an intracellularly cleavable moiety. When A is "nothing," the carbon atoms adjacent to A, on each side, are connected by a single bond, thus giving a five membered ring.

[0014] As used herein, "alkyl" refers to a saturated aliphatic hydrocarbon radical including straight chain and branched chain groups of 1 to 20 carbon atoms (whenever a numerical range; e.g. "1-20", is stated herein, it means that the group, in this case the alkyl group, may contain 1 carbon atom, 2 carbon atoms, 3 carbon atoms, etc. up to and including

20 carbon atoms). Alkyl groups containing from 1 to 4 carbon atoms are referred to as lower alkyl groups. More preferably, an alkyl group is a medium size alkyl having 1 to 10 carbon atoms e.g., methyl, ethyl, propyl, 2-propyl, n-butyl, iso-butyl, tert-butyl, pentyl, and the like. Most preferably, it is a lower alkyl having 1 to 4 carbon atoms e.g., methyl, ethyl, propyl, 2-propyl, n-butyl, iso-butyl, or tert-butyl, and the like.

[0015] As used herein "cycloalkyl" refers to a 3 to 8 member all-carbon monocyclic ring, an all-carbon 5-member/6-member or 6-member/6-member fused bicyclic ring or a multicyclic fused ring (a "fused" ring system means that each ring in the system shares an adjacent pair of carbon atoms with each other ring in the system) group wherein one or more of the rings may contain one or more double bonds but none of the rings has a completely conjugated pi-electron system. Examples, without limitation, of cycloalkyl groups are cyclopropane, cyclobutane, cyclopentane, cyclopentene, cyclohexane, cyclohexadiene, adamantane, cycloheptane, cycloheptatriene, and the like. A cycloalkyl group may be substituted or unsubstituted.

[0016] In another preferred embodiment, the intracellularly cleavable moiety is a hydrazone.

[0017] In a preferred embodiment, the mAb is a mAb that targets tumor-associated antigens. Tumor-associated antigens are defined as antigens expressed by tumor cells, or their vasculature, in a higher amount than in normal cells, wherein the normal cells are vital to cellular functions essential for the patient to survive. Tumor-associated antigens may also be antigens associated with different normal cells, such as lineage antigens in hematopoietic cells, B-cells, T-cells or myeloid cells, whereby a patient can survive with a transient, selective decrease of said normal cells, while the malignant cells expressing the same antigen(s) are sufficiently destroyed to relieve the patient of symptoms and also improve the patient's condition. The mAb may also be reactive with an antigen associated with hematologic malignancies

[0018] In yet another embodiment, the mAb is selected from the group of B-cell, T-cell, myeloid-cell, and other hematopoietic cell-associated antigens, such as CD19, CD20, CD21, CD22, CD23 in B-cells; CD33, CD45, and CD66 in myeloid cells; IL-2 (TAC or CD25) in T-cells; MUC1, tenascin, CD74, HLA-DR, CD80 in diverse hematopoietic tumor types; CEA, CSAp, MUC1, MUC2, MUC3, MUC4, PAM4, EGP-1, EGP-2, AFP, HCG, HER2/neu, EGFR, VEGF, PlGF, Le(y), carbonic anhydrase IX, PAP, PSMA, MAGE, S100, tenascin, and TAG-72 in various carcinomas, tenascin in gliomas, and antigens expressed by the vasculature and endothelial cells, as well as the supportive stroma, of certain tumors.

In still another preferred embodiment, the mAb is selected from the group consisting of LL1 (anti-CD74), LL2 (anti-CD22), hA20 and rituximab (anti-CD20), M195 (anti-CD33), RS7 (anti-epithelial glycoprotein-1 (EGP-1)), 17-1A (anti-EGP-2), PAM-4, BrE3, and KC4 (all anti-MUC1), MN-14 (anti-carcinoembryonic antigen (CEA)), Mu-9 (anti-colon-specific antigen-p), Immu 31 (an anti-alpha-fetoprotein), anti-TAG-72 (e.g., CC49) anti-Tn, J591 (anti-PSMA), BC-2 (an anti-tenascin antibody) and G250 (an anti-carbonic anhydrase IX mAb). Other useful antigens that may be targeted using these conjugates include HER-2/*neu*, CD19, CD20 (e.g., C2B8, hA20, cA20, 1F5 Mabs) CD21, CD23, CD33, CD40, CD80, alpha-fetoprotein (AFP), VEGF, EGF receptor, PlGF (placenta growth factor), ILGF-1 (insulin-like growth factor-1), MUC1, MUC2, MUC3, MUC4, PSMA, gangliosides, HCG, EGP-2 (e.g., 17-1A), CD37, HLA-DR, CD30, Ia, Ii, A3, A33, Ep-CAM, KS-1, Le(y), S100, PSA, tenascin, folate receptor, Thomas-Friedenreich antigens, tumor necrosis antigens, tumor angiogenesis antigens, Ga 733, IL-2 (CD25), T101, MAGE, CD66, CEA, NCA95, NCA90 or a combination thereof.

[0019] In an especially preferred embodiment, the targeting mAb is directed against a surface antigen which is then rapidly internalized with the antibody.

[0020] In an especially preferred embodiment the targeting mAb is directed against the CD74 antigen.

[0021] In yet another preferred embodiment, the linker is a 4-[N-maleimidomethyl]cyclohexane-1-carboxylhydrazide radical.

[0022] Also described are processes for the preparation of the compositions of the invention, together with methods-of-use of the said compositions.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] FIG 1 is a representative size-exclusion HPLC trace of an anthracycline-antibody conjugate prepared using the methods described.

[0024] FIG 2 illustrates the *in vitro* efficacy of the DOX-LL1 conjugate against Burkitt lymphoma Raji cells, versus a DOX conjugate of the non-targeting MN-14 antibody at a concentration of drug-mAb conjugate of 1 μg/mL. The DOX-LL1 conjugate shows a three-order of magnitude difference in the fraction of surviving cells, in comparison to the DOX-MN-14 conjugate.

[0025] FIG 3 is illustrates the efficacy of a single 100 μg dose of 2-PDOX-RS7 conjugate in the DU145 prostate xenograft model in nude mice.

[0026] FIG 4 illustrates the efficacy of single doses of 2-PDOX- and DOX-conjugates of the LL1 antibody in the aggressive RAJI/SCID mouse systemic tumor model. Animals were injected i.v. with Raji B-cell lymphoma cells, and treated five days later with the conjugates designated in the figure.

[0027] FIG 5 illustrates the efficacy of a single dose of 2-PDOX-LL1 antibody in the aggressive RAJI/SCID mouse systemic tumor model, compared to untreated controls given no conjugate, or a group of animals given the non-targeting control conjugate, 2-PDOX-MN-14.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0028] As used herein, "a" or "an" means "one or more" unless otherwise specified.

Introduction

[0029] Chemotherapeutic drugs, such as those discussed above, can be coupled to antibodies by several methods to form a mAb-drug conjugate. For example, the chemotherapeutic drugs may be attached to the mAb, or fragments thereof, after reduction of the mAb inter-chain disulfide bonds. This approach generates an average of eight-to-ten (depending on IgG type) free thiol groups per molecule of antibody, and does so in a reproducible manner at the limiting levels of thiol used in the reduction reaction. This method of attachment of the chemotherapeutic drugs is advantageous for the following reasons: first, the attached chemotherapeutic drugs are placed in an internal or semi-internal site on the mAb, or fragments thereof, which is not exposed on hydrophilic lysine residues. This serves to keep them more stable due to the more hydrophobic areas of the mAb, where the chemotherapeutic drugs are placed. Second, such a site does not alter the overall charge of the mAb, or fragments thereof. Third, placement on internal thiols is less likely to interfere in the ADCC and complement actions that are particularly important when naked versions of the mAb are used. Thus, the attachment site is chosen to be non-interfering, such that ADCC and complement fixation, can be complementary to the mAbs, or the mAb fragments, role as a drug delivery vehicle. Fourth, placement at the internal thiol positions is less likely to lead to an immune response to the chemotherapeutic drugs, compared to placement of a multitude of chemotherapeutic drugs molecules on exposed lysine groups. In some embodiments, the overall electric charge of the antibody in the Ab-drug conjugate is not changed as compared to the charge of the antibody prior to the coupling. This is because

no lysine residues are used in the conjugation reaction, and therefore no free, positive amino groups are modified to form, for example, neutral amide bonds.

Antibodies

[0030] An antibody, as described herein, refers to a full-length (i.e., naturally occurring or formed by normal immunoglobulin gene fragment recombinatorial processes) immunoglobulin molecule (*e.g.*, an IgG antibody) or an immunologically active (i.e., specifically binding) portion of an immunoglobulin molecule, like an antibody fragment.

[0031] An antibody fragment is a portion of an antibody such as $F(ab')_2$, $F(ab)_2$, Fab', Fab, Fv, scFv (single chain Fv) and the like. Regardless of structure, an antibody fragment binds with the same antigen that is recognized by the intact antibody and it therefore, an antigen-binding fragment of the antibody of which it is a portion.

[0032] The term "antibody fragment" also includes any synthetic or genetically engineered protein that acts like an antibody by binding to a specific antigen to form a complex. For example, antibody fragments include isolated fragments consisting of the variable regions, such as the "Fv" fragments consisting of the variable regions of the heavy and light chains, recombinant single chain polypeptide molecules in which light and heavy variable regions are connected by a peptide linker ("scFv proteins"), and minimal recognition units consisting of the amino acid residues that mimic the hypervariable region. The Fv fragments may be constructed in different ways as to yield multivalent and/or multispecific binding forms. Multivalent binding forms react with more than one binding site against the specific epitope, whereas multispecific forms bind more than one epitope (either of the antigen or even against the specific antigen and a different antigen).

[0033] As used herein, the term antibody fusion protein is a recombinantly-produced antigen-binding molecule in which two or more of the same or different natural antibody, single-chain antibody or antibody fragment segments with the same or different specificities are linked. A fusion protein comprises at least one specific binding site.

[0034] Valency of the fusion protein indicates the total number of binding arms or sites the fusion protein has to antigen(s) or epitope(s); i.e., monovalent, bivalent, trivalent or mutlivalent. The multivalency of the antibody fusion protein means that it can take advantage of multiple interactions in binding to an antigen, thus increasing the avidity of binding to the antigen, or to different antigens. Specificity indicates how many different types of antigen or epitope an antibody fusion protein is able to bind; i.e., monospecific, bispecific, trispecific, multispecific. Using these definitions, a natural antibody, e.g., an IgG,

is bivalent because it has two binding arms but is monospecific because it binds to one type of antigen or epitope. A monospecific, multivalent fusion protein has more than one binding site for the same antigen or epitope. For example, a monospecific diabody is a fusion protein with two binding sites reactive with the same antigen. The fusion protein may comprise a multivalent or multispecific combination of different antibody components or multiple copies of the same antibody component.

[0035] In a preferred embodiment of the present invention, antibodies, such as monoclonal antibodies (mAbs), are used that recognize or bind to markers or tumor-associated antigens that are expressed at high levels on target cells and that are expressed predominantly or only on diseased cells versus normal tissues, and antibodies that internalize rapidly. Antibodies useful within the scope of the present invention include antibodies against tumor-associated antigens, such as antibodies with properties as described above (and show distinguishing properties of different levels of internalization into cells and microorganisms), and contemplate the use of, but are not limited to, in cancer, the following mAbs: LL1 (anti-CD74), LL2 (anti-CD22), M195 (anti-CD33), MN3 (anti-NCA90), RS7 (anti-epithelial glycoprotein-1(EGP-1)), PAM-4, BrE3 and KC4 (all anti-MUC1), MN-14 (anti-carcinoembryonic antigen (CEA)), Mu-9 (anti-colon-specific antigen-p), Immu 31 (an anti-alpha-fetoprotein), anti-TAG-72 (e.g., CC49), anti-Tn, J591 (anti-PSMA), M195 (anti-CD33) and G250 (an anti-carbonic anhydrase IX mAb). Other useful antigens and different epitopes of such antigens that may be targeted using these conjugates include HER-2/*neu*, , CD19, CD20 (e.g., C2B8, hA20, 1F5 Mabs) CD21, CD23, CD25, CD30, CD33, CD37, CD40, CD74, CD80, alpha-fetoprotein (AFP), VEGF, EGF receptor, PlGF, MUC1, MUC2, MUC3, MUC4, PSMA, PAP, carbonic anhydrase IX, TAG-72, GD2, GD3, , HCG, EGP-2 (e.g., 17-1A), , HLA-DR, CD30, Ia, A3, A33, Ep-CAM, KS-1, Le(y), S100, PSA, tenascin,, folate receptor, Tn or Thomas-Friedenreich antigens, tumor necrosis antigens, tumor angiogenesis antigens, Ga 733, , T101, MAGE, or a combination thereof. A number of the aforementioned antigens are disclosed in U.S. Provisional Application Serial No. 60/426,379, entitled "Use of Multi-specific, Non-covalent Complexes for Targeted Delivery of Therapeutics," filed November 15, 2002.

[0036] In another preferred embodiment of the present invention, antibodies are used that internalize rapidly and are then re-expressed on cell surfaces, enabling continual uptake and accretion of circulating antibody-chemotherapeutic drug conjugate by the cell. In a preferred embodiment, the drug is anthracycline and the antibody-anthracyline conjugate is internalized into target cells and then re-expressed on the cell surface. An example of a most-

preferred antibody/antigen pair is LL1 and CD74 (invariant chain, class II-specific chaperone, Ii). The CD74 antigen is highly expressed on B cell lymphomas, certain T cell lymphomas, melanomas and certain other cancers (Ong et al., *Immunology 98*:296-302 (1999)).

[0037] In a preferred embodiment the antibodies that are used in the treatment of human disease are human or humanized (CDR-grafted into a human framework) versions of antibodies; although murine, chimeric and primatized versions of antibodies can be used. For veterinary uses, the same-species IgG would likely be the most effective vector, although cross-species IgGs would remain useful, such as use of murine antibodies in dogs (e.g., L243 anti-HLA-DR mAb for treating canine lymphoma). Same species immunoglobulin (IgG)s molecules as delivery agents are mostly preferred to minimize immune responses. This is particularly important when considering repeat treatments. For humans, a human or humanized IgG antibody is less likely to generate an anti-IgG immune response from patients. Targeting an internalizing antigen, antibodies such as hLL1 and hLL2 rapidly internalize after binding to target cells, which means that the conjugated chemotherapeutic drug is rapidly internalized into cells.

[0038] An immunomodulator, such as a cytokine can also be conjugated to the monoclonal antibody-anthracycline drug, or can be administered unconjugated to the chimeric, humanized or human monoclonal antibody-anthracycline drug conjugate of the preferred embodiments of the present invention. The immunomodulator can be administered before, concurrently or after administration of the monoclonal antibody-anthracyline drug conjugate of the preferred embodiments of the present invention. The immunomodulator can also be conjugated to a hybrid antibody consisting of one or more antibodies binding to different antigens. Such an antigen may also be an immunomodulator. For example, CD40 or other immunomodulators can be administered in combination with anti-CSAp or anti-CSAp/non-CSAp antibody combination either together, before or after the antibody combinations are administered. The monoclonal antibody-anthracyline drug conjugate can also be used in combination with, or conjugated to, as a fusion protein, such as against CD40.

[0039] As used herein, the term "immunomodulator" includes cytokines, stem cell growth factors, lymphotoxins, such as tumor necrosis factor (TNF), and hematopoietic factors, such as interleukins (e.g., interleukin-1 (IL-1), IL-2, IL-3, IL-6, IL-10, IL-12, IL-18, and IL-21), colony stimulating factors (e.g., granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF)), interferons (e.g.,

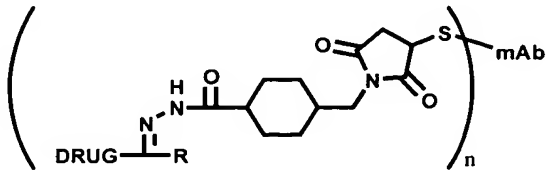
interferons-α, -β and -γ), the stem cell growth factor designated "S1 factor," erythropoietin and thrombopoietin. Examples of suitable immunomodulator moieties include IL-2, IL-6, IL-10, IL-12, IL-18, IL-21, interferon-γ, TNF-α, and the like.

[0040] An immunomodulator is a therapeutic agent as defined in the present invention that when present, alters, suppresses or stimulates the body's immune system. Typically, the immunomodulator useful in the present invention stimulates immune cells to proliferate or become activated in an immune response cascade, such as macrophages, B-cells, and/or T-cells. An example of an immunomodulator as described herein is a cytokine, which is a soluble small protein of approximately 5-20 kDs that are released by one cell population (e.g., primed T-lymphocytes) on contact with specific antigens, and which act as intercellular mediators between cells. As the skilled artisan will understand, examples of cytokines include lymphokines, monokines, interleukins, and several related signalling molecules, such as tumor necrosis factor (TNF) and interferons. Chemokines are a subset of cytokines. Certain interleukins and interferons are examples of cytokines that stimulate T cell or other immune cell proliferation.

[0041] In a preferred embodiment of the present invention, the immunomodulator enhances the effectiveness of the anthracycline drug-antibody conjugate, and in some instances by stimulator effector cells of the host.

Antibody-chemotherapeutic drug conjugates

[0042] The present invention is directed to a conjugate of an anthracycline drug and an antibody, wherein the anthracycline drug and the antibody are linked via a linker comprising a hydrazide and a maleimide. The linker preferably is 4-(N-maleimidomethyl)cyclohexane-1-carboxyl hydrazide. The conjugate preferably has the formula:



wherein n is 6 to 10.

[0043] Further, the antibody is directed against or recognizes a tumor-associated antigen. The antibody may be a monoclonal antibody, an antigen-binding fragment thereof or an antibody fusion protein. The antibody fusion protein may be multivalent and/or

multispecific. The antibody fusion protein in the conjugate may comprise two or more of the same or different natural or synthetic antibody, single-chain antibody or antibody fragment segments with the same or different specificities. The antibody or antibody fragment of the fusion protein can be selected from the group consisting of LL1, LL2, M195, MN-3, RS7, 17-1A, RS11, PAM-4, KC4, BrE3, MN-14, Mu-9, Immu 31, CC49,, Tn antibody, J591, Le(y) antibody and G250.

[0044] This tumor-associated antigen may be targeted by an internalizing antibody. The conjugate is useful for targeting carcinomas, sarcomas, lymphomas, leukemias, gliomas or skin cancers, such as melanomas. The tumor-associated antigen preferably is selected from the group consisting of CD74, CD22, EPG-1, CEA, colon-specific antigen-p mucin (CSAp), carbonic anhydrase IX, HER-2/*neu*, , CD19, CD20, CD21, CD23, CD25, CD30, CD33, CD40, CD45, CD66, NCA90, NCA95, CD80, alpha-fetoprotein (AFP), VEGF, EGF receptor, PlGF, MUC1, MUC2, MUC3, MUC4, PSMA, GD2, GD3 gangliosides, HCG, EGP-2, CD37, HLA-D-DR, CD30, Ia, Ii, A3, A33, Ep-CAM, KS-1, Le(y), S100, PSA, tenascin, folate receptor, Tn and Thomas-Friedenreich antigens, tumor necrosis antigens, tumor angiogenesis antigens, Ga 733, IL-2, , MAGE, and a combination thereof. More preferably the tumor-associated antigen is selected from the group consisting of CD74, CD19, CD20, CD22, CD33, EPG-1, MUC1, CEA and AFP. These tumor-associated antigens may be lineage antigens (CDs) of B-cells, T-cells, myeloid cells, or antigens associated with hematologic malignancies.

[0045] The antibody portion of the conjugate can be murine, chimeric, primatized, humanized, or human. The antibody may be an intact immunoglobulin or an antigen-binding fragment thereof, such as an IgG or a fragment thereof. Preferably, the antibody is directed against B-cells, such as an antigen selected from the group consisting of CD19, CD20, CD21, CD22, CD23, CD30, CD37, CD40, CD52, CD74, CD80, and HLA-DR. The antibody, antigen-binding fragment thereof or fusion protein, preferably is selected from the group of LL1, LL2, L243, C2B8, A20, MN-3, M195, MN-14, anti-AFP, Mu-9, PAM-4, RS7, RS11 and 17-1A. More preferably, the antibody is LL1, LL2, L243, C2B8, or hA20. Additionally, the antibody is linked to the drug via a linker which is attached to a reduced disulfide bond on the antibody, which may be an interchain disulfide bond on the antibody.

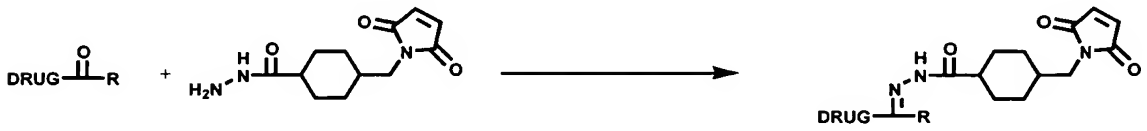
[0046] The anthracycline drug portion of the conjugate is selected from the group consisting of daunorubicin, doxorubicin, epirubicin, 2-pyrrolinodoxorubicin, morpholino-doxorubicin, and cyanomorpholino-doxorubicin. Further, the anthracycline drug can be linked to the antibody through the 13-keto moiety. Preferably, there are 6 - 10 molecules of

anthracycline drug per molecule of antibody. Additionally, the antibody-anthracycline conjugate is internalized into target cells, and the antigen is then re-expressed on the cell surface.

[0047] The present invention is directed to a process for producing the conjugate described herein, wherein the linker is first conjugated to the anthracycline drug, thereby producing an anthracycline drug-linker conjugate, and wherein the anthracycline drug-linker conjugate is subsequently conjugated to a thiol-reduced monoclonal antibody or antibody fragment. The anthracycline drug-linker conjugate may be purified prior to conjugation to the thiol-reduced monoclonal antibody or antibody fragment but it is not necessary to do so. Thus, preferably there is no need to purify the anthracycline drug-linker conjugate prior to conjugation to the thiol-reduced monoclonal antibody or antibody fragment. The process for preparing the conjugate should be such that the secondary reactive functional groups on the anthracycline drug are not compromised. Additionally, the process for preparing the conjugate should not compromise the alkylating groups on the anthracycline drugs. The anthracycline drug in the conjugate preferably is 2-pyrrolino-doxorubicin, morpholino-doxorubicin or cyanomorpholino-doxorubicin.

[0048] The chemotherapeutic drug molecules are separately activated for conjugation to the antibody such that they contain a free maleimide group, specific for thiol reaction at neutral pH. When the chemotherapeutic drug bears a reactive ketone, the ketone can be converted to hydrazone using the commercially available linker 4-[N-maleimidomethyl]cyclohexane-1-carboxylhydrazide (M_2C_2H; Pierce Chemical Co., Rockford, IL) [also supplied as the trifluoroacetate salt by Molecular Biosciences, Inc., Boulder, CO] as shown in Scheme I, below.

[0049] In Scheme I, the DRUG is a chemotherapeutic drug, preferably an anthracycline drug and the R group is either a hydrogen atom or a C_1-C_6 alkyl group optionally substituted with a hydroxyl group (-OH).

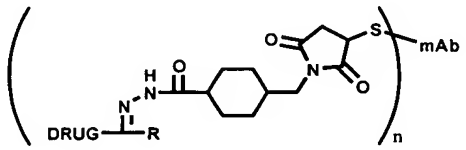


Scheme I

[0050] While not being bound by theory, the linker M_2C_2H is thought to be a particularly useful linker in the context of the preferred embodiments of the present invention for two reasons. First, the cyclohexyl group in the linker is thought to stabilize the hydrazone

functionality. It is important that the hydrazone linkage used is substantially stable to serum conditions, and the cyclohexyl group proximal to the formed hydrazone results in a more stable hydrazone bond in comparison to a more standard straight-chain alkyl group. Second, the hydrazone produced from the reaction of the ketone with this carboxylhydrazide is cleaved once the chemotherapeutic drug-mAb conjugate is internalized into the cell.

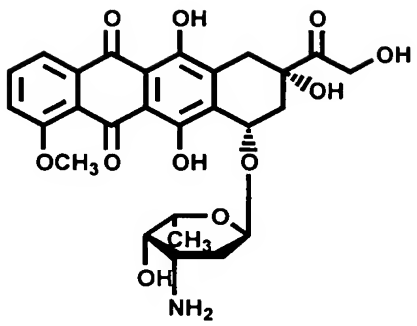
[0051] The maleimide-substituted chemotherapeutic drugs, in slight excess (1 to 5 fold molar) to available thiol groups on the reduced mAb are mixed in an aqueous solution with the reduced mAb. The reaction is performed at neutral, near-neutral or below neutral pH, preferably from about pH 4 to about pH 7. The components are allowed to react for a short reaction time of from about 5 to about 30 minutes. The skilled artisan would recognize, however, that the reaction conditions may be optimized with respect to reaction time and pH. The chemotherapeutic drug-mAb conjugate, shown schematically below (wherein n is an integer from 1 to 10, preferably from 1 to 8), is then separated from chemotherapeutic drug and other buffer components by chromatography through size-exclusion and hydrophobic interaction chromatography columns. In a preferred embodiment, the drug is an anthracycline and n is an integer from 6 - 10.



Chemotherapeutic drug-mAb conjugate

[0052] The above conditions are optimal in the case of 2-PDOX. The reaction conditions are optimal since they ensure that only the freely generated thiol groups of the mAb react with the maleimide-activated chemotherapeutic drug, while the enamine of 2-PDOX is not impinged by the reaction conditions. It is surprising that the thiol-maleimide coupling can be carried out in the presence of an alkylatable group, as exemplified here by the enamine group.

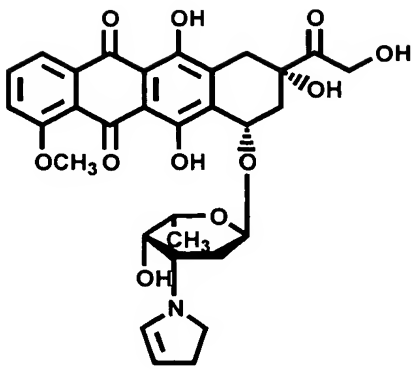
[0053] In a preferred embodiment of the present invention, the chemotherapeutic drugs that are used are anthracycline drugs. These drugs comprise a large class of derivatives typified by one of the original members of the group, doxorubicin (DOX, shown below), and its isomer, epirubicin.



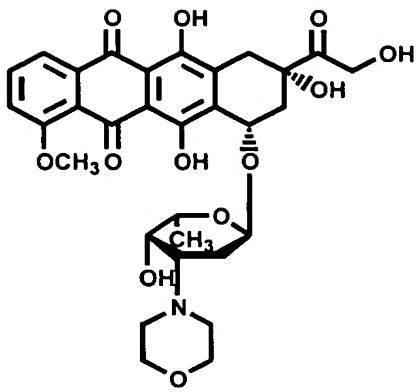
DOX

[0054] Both doxorubicin and epirubicin are widely used in cancer therapy. In another preferred embodiment of the present invention the chemotherapeutic drugs include analogs of the highly toxic 2-PDOX, namely, morpholino- and cyanomorpholino-doxorubicin (morpholino-DOX and cyanomorpholino DOX, respectively). In another embodiment the chemotherapeutic drugs include daunorubicin.

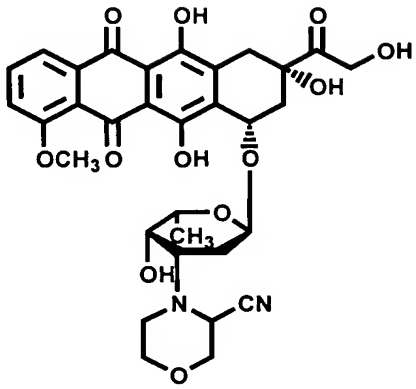
[0055] The skilled artisan will recognize that the anthracycline drugs of the preferred embodiments of the present invention contain a number of reactive groups, which may be referred go as secondary reactive functional groups, that may require protection with protective groups well known in the art prior to conjugation of the drug with the linker and/or prior to conjugation of the drug-linker conjugate and the mAb; protection may be necessary so as to not compromise the integrity of the reactive groups. *See* Greene and Wuts, *Protective Groups in Organic Synthesis* (John Wiley & Sons 2d ed. 1991. The reactive groups include the carbonyl groups in the anthraquinone core of the anthracycline drugs; groups which, under certain conditions, may be react with a nucleophile. Other reactive groups include the various alcohol groups that are located throughout the anthracycline drug molecules; groups, which under certain conditions may react with electrophiles. Lastly, other reactive groups include the amine group present in DOX and the enamine group in 2-PDOX; both of which may react with an electrophile. In the case of anthracycline drugs bearing an alkylating group (e.g., the enamine of 2-PDOX), it may be necessary to control the reaction conditions such that the integrity of the alkylating group is not compromised.



2-PDOX



Morpholino-DOX



Cyanomorpholino-DOX

[0056] Within the anthracycline drug class, individual drugs, of toxicities varying over a 1-10,000 fold range (3-4 order-of-magnitude) range, can be interchanged on the basis of their varying toxicities, in order to generate more or less toxic immunoconjugates. Anthracyclines can exert their toxic effect on target cells by several mechanisms, including inhibition of DNA topoisomerase 2 (top 2), intercalation into DNA, redox reactions and binding to certain intracellular or membrane proteins. Additionally, analogs can be designed that have additional mechanisms of cell killing, such as a potential to be alkylated. Exemplary analogs are anthracylines bearing an alkylating moiety, as in the case of the 2-PDOX analog. In this instance, the alkylating moiety is an enamine group. In the 2-PDOX analog, the enamine group in the pyrrolino- ring is highly reactive to nucleophiles under physiologic conditions.

Pharmaceutical Compositions and Methods of Administrations

[0057] Some embodiments of the present invention relate to a pharmaceutical composition comprising the mAb-drug conjugate of the present invention and a

pharmaceutically acceptable carrier or excipient. By "pharmaceutically acceptable carrier" is intended, but not limited to, a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type known to persons skilled in the art. Diluents, such as polyols, polyethylene glycol and dextrans, may be used to increase the biological half-life of the conjugate.

[0058] The present invention also is directed to a method for treating disease in a mammal comprising administering a conjugate of an antibody and an anthracycline drug as described herin. The present method also comprises administering the antibody-anthracycline conjugate described herein in all of it permutations preceded by, concomitantly with, or subsequent to other standard therapies, wherein said standard therapy is selected from the group consisting of radiotherapy, surgery and chemotherapy.

[0059] The present invention is intended to encompass a method for treating disease in a mammal comprising administering two or more conjugates of an antibody and an anthracycline drug that target different antigens or different epitopes of the same antigen on the same diseased cells. Additionally the present invention is intended to encompass a method for treating disease in a mammal comprising administering a conjugate of an antibody and an anthracycline drug preceded by, concomitantly with, or subsequent to a second antibody-based treatment, such that the second antibody in the second antibody-based treatment targets a different antigen or a different epitope on the same antigen on diseased cells than the antibody in the conjugate.

[0060] In some embodiments, the mAb-drug conjugate alone or a pharmaceutical composition comprising the mAb-drug conjugate of the present invention and a pharmaceutically acceptable carrier or excipient may be used in a method of treating a subject, comprising administering a therapeutically effective amount of the mAb-drug conjugate of the present invention to a subject.

[0061] In preferred embodiments, the subject is a mammal. Exemplary mammals include human, pig, sheep, goat, horse, mouse, dog, cat, cow, etc. Diseases that may be treated with the mAb-drug conjugate of the present invention include cancer, such as cancer of the skin, head and neck, lung, breast, prostate, ovaries, endometrium, cervix, colon, rectum, bladder, brain, stomach, pancreas, lymphatic system may be treated. Patients suffering from B- or T-cell cancer, non-Hodgkin's lymphoma, Hodgkin's disease, lymphatic or myeloid leukemias, multiple myeloma, sarcoma and melanoma may be treated by administration of a therapeutic amount of the mAb-drug conjugate of the present invention.

[0062] The mAb-drug conjugate of the present invention may be administered intravenously, intra-peritoneally, intra-arterially, intra-thecally, intra-vesically, or intratumorally. The conjugate may be given as a bolus or as an infusion on a repeat and/or a cyclical basis. The infusion may be repeated for one or more times depending on the dose of drug and tolerability of the conjugate in terms of side effects and is determined by the managing physician. One of ordinary skill will appreciate that effective amounts of the mAb-drug conjugate of the invention can be determined empirically. The agents can be administered to a subject, in need of treatment of cancer, as pharmaceutical compositions in combination with one or more pharmaceutically acceptable excipients. It will be understood that, when administered to a human patient, the total daily usage of the agents or composition of the present invention will be decided by the attending physician within the scope of sound medical judgement. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors: the type and degree of the cellular response to be achieved; activity of the specific mAb-drug conjugate or composition employed; the specific mAb-drug conjugate or composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the agent; the duration of the treatment; drugs used in combination or coincidental with the specific agent; and like factors well known in the medical arts. For example, it is well within the skill of the art to start doses of the agents at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosages until the desired effect is achieved.

[0063] In a preferred embodiment of the present invention, the antibody-anthracycline conjugate is administered preceded by, concomitantly with, or subsequent to other standard therapies including radiotherapy, surgery or chemotherapy.

[0064] In another preferred embodiment, two or more conjugates of an antibody and an anthracycline drug are administered which conjugates target different antigens or different epitopes of the same antigen on the same diseased cells. In yet another preferred embodiment, a conjugate of an antibody and an anthracycline drug is administered, preceded by, concomitantly with, or subsequent to another antibody-based treatment. This additional antibody-based treatment may include the administration of two or more antibody-based treatments, to include naked therapy, where the antibody is administered alone or in combination with another therapeutic agent that is administered either conjugated or unconjugated to the antibody. The conjugation may utilize the presently disclosed linker or another type linker. When two antibody-based treatments are administered, these treatment

are such that whichever antibody is administered second targets a different antigen or a different epitope on the same antigen on diseased cells. The second antibody could also be conjugated with another (different) drug or with a therapeutic isotope, thus providing an antibody-based combination therapy. It is also appreciated that this therapy can be combined, with administration before, simultaneously, or after with cytokines that either enhance the antitumor effects or prevent or mitigate the myelosuppressive effects of the therapeutic conjugates.

[0065] Each of the above identified methods of treatment may additionally include the administration of one or more immunomodulators. These immunomodulators may be selected from the group consisting of interferons, cytokines, stem cell growth factors, colony-stimulating factors, lymphotoxins and other hematopoietic factors. The interferon is preferably α-interferon, β-inerferon or γ-interferon and the hematopoietic factors may be selected from the group consisting of erythropoietin, thrombopoietin, interleukins (ILs), colony stimulating factors (CSF), granulocyte macrophage-colony stimulating factor (GM-CSF). The interleukin may be selected from the group consisting of IL-1, IL-2, IL-3, IL-6, IL-10, IL-12, IL-18, and IL-21. The immunomodulator or heamatopoietic factor may administered before, during, or after immunconjugate therapy. The immunomodulator is administered to enhance the effectiveness of the administered conjugate of the present invention.

Kits

[0066] The preferred embodiments of the present invention also contemplate kits comprising a conjugate of a monoclonal antibody and an anthracycline drug in a suitable container. The conjugate preferably includes a linker comprising a hydrazide and a malemide. The monoclonal antibody-anthracycline drug conjugate is provided in a sterile container in liquid, frozen or lyophilized form. The monoclonal antibody-anthracycline drug conjugate can be diluted or reconstituted prior to administration to a patient in need thereof.

[0067] In a further embodiment, the conjugate of an anthracycline drug and an antibody, wherein the anthracycline drug and the antibody are linked via a linker comprising a hydrazide and a maleimide and wherein at least one immunomodulator is further conjugated to the antibody. The conjugate can then be administered to patients in need of therapy as described herein for the conjugate alone or in combination other therapies.

[0068] The present invention is illustrated by the following examples, without limiting the scope of the invention.

EXAMPLES

General

[0069] 2-pyrrolino-doxorubicin was prepared using a modified method, based on the original description of Nagy *et al.* (*Proc. Natl. Acad. Sci, U.S.A. 93*:2464-2469 (1996)). Morpholino-DOX and cyanomorpholino-DOX were both synthesized from doxorubicin using published methods (Acton *et al., J. Med. Chem. 27*:638-645 (1984)).

Example 1: Synthesis of 2-PDOX

[0070] Synthesis of 2-pyrrolino-doxorubicin (2-PDOX): 4-iodobutyraldehyde: 2-(3-chloropropyl)-1,3-dioxolane (1.3 mL; 10 mM) was dissolved in 200 mL of acetone containing 30 g of sodium iodide (200 mmol; 20-fold excess). The solution is refluxed for 24 h and then evaporated to dryness. The crude mixture is used in the next reaction. Doxorubicin hydrochloride (550 mg, 946 μmol) is dissolved in 6.5 mL of DMF and 3.86 g (19.48 mmol, 20-fold excess) of 4-iodobutyraldehyde is added followed by 500 μL of N,N-diisopropylethylamine (DIPEA). After five minutes the material is purified by reverse-phase HPLC on a Waters NovaPak C-18 column using a gradient elution. The gradient consisted of 90:10 eluent A to 70:30 eluent B at 75 mL per minute, over 40 minutes, where eluent A is 0.1% trifluoroacetic acid (TFA) and eluent B is 90% acetonitrile containing 0.1% TFA. The identity of the product was confirmed by electrospray mass spectrometry $M + H^+ = 596$.

Example 2: Conjugation of 2-PDOX to the anti-CD22 antibody humanized LL2 (hLL2)

[0071] a) Activation of 2-PDOX: 2-PDOX (5.95 mg; 1×10^{-5} mol) is mixed with a molar equivalent of the commercially available linker 4-[N-maleimidomethyl]cyclohexane-1-carboxylhydrazide (M_2C_2H; Pierce Chemical Co., Rockford, IL) (2.88 mg; 1×10^{-5} mol) in 0.5 mL of dimethylsulfoxide (DMSO). The reaction mixture is heated at 50-60° C under reduced pressure for thirty minutes. The desired product is purified by preparative RP-HPLC, using a gradient consisting of 0.3 % ammonium acetate and 0.3 % ammonium acetate in 90% acetonitrile, pH 4.4, to separate the desired product from most of the unreacted 2-PDOX (eluting ~ 0.5 minute earlier) and from unreacted M_2C_2H (eluting much earlier). The amount recovered is estimated by reference to the UV absorbance level of the sample (496 nm), versus a standard solution of 2-PDOX in acetonitrile/ammonium acetate buffer. The

maleimide-activated 2-PDOX is frozen and lyophilized, if not used immediately. It is taken up in the minimum amount of DMSO when needed for future reaction with antibodies.

[0072] b) Reduction of hLL2 IgG: A 1-mL sample of LL2 antibody (8-12 mg/mL) at 4°C is treated with 100 μL of 1.8 M Tris HCl buffer, followed by three μL of 2-mercaptoethanol. The reduction reaction is allowed to proceed for 10 minutes, and the reduced antibody is purified through two consecutive spin-columns of G-50-80 Sephadex equilibrated in 0.1 M sodium acetate, pH 5.5, containing 1 mM EDTA as anti-oxidant. The product is assayed by UV absorbance at 280 nm, and by Ellman reaction with detection at 410 nm, to determine the number of thiol groups per mole of antibody. These reduction conditions result in the production of approximately 8-12 thiol groups per antibody, corresponding to complete reduction of the antibody's inter-chain disulfide bonds.

[0073] c) Conjugation of Activated 2-PDOX to reduced hLL2: The thiol-reduced antibody from b), above, is treated with maleimido-activated 2-PDOX, without allowing the final concentration of DMSO to go above 25% in the aqueous/DMSO mixture. After reaction for 15 minutes at 4° C, the desired product is obtained free of unreacted maleimido-DOX by elution through a G-50-80 spin-column, equilibrated in 0.2 M ammonium acetate, pH 4.4, followed by percolation through a column of SM-2 Bio-Beads equilibrated in the same buffer. The product is analyzed by UV scan at 280 and 496 nm, and the molar ratio of 2-PDOX to mAb is estimated thereby. The absolute 2-PDOX-to-MAb ratio is determined by MALDI-TOF mass spectral analysis. Both UV and MS analyses indicate that a substitution ratio of 7-8 units of 2-PDOX per mole of hLL2 antibody, is obtained under this set of reaction conditions. Upon analysis by size-exclusion HPLC (GF-250 column, Bio-Rad, Hercules CA) run at 1 mL/minute in 0.2 M acetate buffer, pH 5.0, with a UV detector set at 496 nm, essentially all the detected peak elutes near the retention time of the LL2 antibody. This indicates that very little free drug is present in the product. Samples of 2-PDOX-hLL2 conjugate are aliquoted into single fractions, typically of 0.1-1.0 mg, and frozen for future use, or, alternatively, they are lyophilized. They are defrosted or reconstituted, as needed, for further testing.

Example 3: Conjugation of 2-PDOX to the anti-CD74 antibody humanized LL1 (hLL1)

[0074] a) Activation of 2-PDOX: 2-PDOX (5.95 mg; 1 x 10^{-5} mol) is mixed with a molar equivalent of the commercially available linker 4-[N-maleimidomethyl]cyclohexane-1-

carboxylhydrazide (M_2C_2H; Pierce Chemical Co., Rockford, IL) (2.88 mg; 1 x 10^{-5} mol) in 0.5 mL of DMSO. The reaction mixture is heated at 50-60° C under reduced pressure for thirty minutes. The desired product is purified by preparative RP-HPLC, using a gradient consisting of 0.3 % ammonium acetate and 0.3 % ammonium acetate in 90% acetonitrile, pH 4.4, to separate the desired product from most of the unreacted 2-PDOX (eluting ~ 0.5 minute earlier) and from unreacted M_2C_2H (eluting much earlier). The amount recovered is estimated by reference to the UV absorbance level of the sample (496 nm), versus a standard solution of 2-PDOX in acetonitrile/ammonium acetate buffer. The maleimide-activated 2-PDOX is frozen and lyophilized, if not used immediately. It is taken up in the minimum amount of dimethylformamide (DMF) or DMSO when needed for future reaction with antibodies.

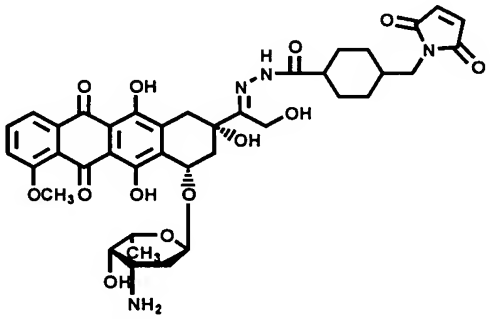
[0075] b) Reduction of hLL1 IgG: A 1-mL sample of hLL1 antibody (8-12 mg/mL) at 4°C is treated with 100 µL of 1.8 M Tris HCl buffer, followed by three µL of 2-mercaptoethanol. The reduction reaction is allowed to proceed for 10 minutes, and the reduced antibody is purified through two consecutive spin-columns of G-50-80 Sephadex equilibrated in 0.1 M sodium acetate, pH 5.5, containing 1 mM EDTA as anti-oxidant. The product is assayed by UV absorbance at 280 nm, and by Ellman reaction with detection at 410 nm, to determine the number of thiol groups per mole of antibody. These reduction conditions result in the production of approximately eight-to-ten thiol groups per antibody, corresponding to complete reduction of the antibody's inter-chain disulfide bonds.

[0076] c) Conjugation of Activated 2-PDOX to reduced hLL1: The thiol-reduced antibody from b), above, is treated with maleimido-activated 2-PDOX from a) above, with the final concentration of DMSO of 15% in the aqueous/DMSO mixture. After reaction for 15 minutes at 4°C, the desired product is obtained free of unreacted maleimido-DOX by elution through a G-50-80 spin-column, equilibrated in 0.2 M ammonium acetate, pH 4.4, followed by percolation through a column of SM-2 Bio-Beads equilibrated in the same buffer. The product is analyzed by UV scan at 280 and 496 nm, and the molar ratio of 2-PDOX to mAb is estimated thereby. The absolute 2-PDOX-to-MAb ratio is determined by MALDI-TOF mass spectral analysis. Both UV and MS analyses indicate that a substitution ratio of 7-8 units of 2-PDOX per mole of hLL1 antibody, is obtained under this set of reaction conditions. Upon analysis by size-exclusion HPLC (GF-250 column, Bio-Rad, Hercules CA) run at 1 mL/minute in 0.2 M acetate buffer, pH 5.0, with a UV detector set at 496 nm, essentially one detected peak elutes near the retention time of the hLL1 antibody. This indicates that very little free or no drug is present in the product. Samples of 2-PDOX-

hLL1 conjugate are aliquoted into single fractions, typically of 0.1-1.0 mg, and frozen for future use, or alternatively they are lyophilized. They are defrosted or reconstituted, as needed, for further testing.

Example 4: Conjugation of DOX to the anti-CD74 antibody hLL1

[0077] a) Activation of DOX: DOX (1×10^{-5} mol) is mixed with a molar equivalent of the commercially available linker 4-[N-maleimidomethyl] cyclohexane-1-carboxylhydrazide (M_2C_2H; Pierce Chemical Co., Rockford, IL) (2.88 mg; 1×10^{-5} mole) in 0.5 mL of DMSO. The reaction mixture is heated at 50-60° C for thirty minutes. The desired intermediate, shown below, is purified by preparative RP-HPLC, using a gradient consisting of 0.3 % ammonium acetate and 0.3 % ammonium acetate in 90% acetonitrile, pH 4.4, to separate the desired product from the unreacted DOX (eluting ~ 0.5 minute earlier) and from unreacted M_2C_2H (eluting much earlier).



[0078] The amount of unreacted DOX is estimated by reference to the UV absorbance level of the sample (496 nm), versus a standard solution of DOX in acetonitrile/ammonium acetate buffer. The maleimide-activated DOX is frozen and lyophilized, if not used immediately. It is taken up in the minimum amount of DMF or DMSO when needed for future reaction with antibodies.

[0079] b) Reduction of hLL1 IgG: A 1-mL sample of hLL1 antibody (10 mg/mL) at 4°C is treated with 100 µL of 1.8 M Tris HCl buffer, followed by three µL of 2-mercaptoethanol. The reduction reaction is allowed to proceed for 10 minutes, and the reduced antibody is purified through two consecutive spin-columns of G-50-80 Sephadex equilibrated in 0.1 M sodium acetate, pH 5.5, containing 1 mM EDTA as anti-oxidant. The product is assayed by UV absorbance at 280 nm, and by Ellman reaction with detection at 410 nm, to determine the number of thiol groups per mole of antibody. These reduction conditions result in the production of approximately eight-to-ten thiol groups per antibody, corresponding to complete reduction of the antibody's inter-chain disulfide bonds.

[0080] c) Conjugation of activated DOX to reduced hLL1: The thiol-reduced antibody from b), above, is treated with maleimido-activated DOX from a) above, with a final concentration of DMSO of 15% in the aqueous/DMSO mixture. After reaction for 15 minutes at 4°C, the desired product is obtained free of unreacted maleimido-DOX by elution through a G-50-80 spin-column, equilibrated in 0.2 M ammonium acetate, pH 4.4, followed by percolation through a column of SM-2 Bio-Beads equilibrated in the same buffer. The product is analyzed by UV scan at 280 and 496 nm, and the molar ratio of DOX to mAb is estimated thereby. The absolute DOX-to-MAb ratio is determined by MALDI-TOF mass spectral analysis. Both UV and MS analyses indicate that a substitution ratio of 7-8 units of DOX per mole of hLL1 antibody, is obtained under this set of reaction conditions. Upon analysis by size-exclusion HPLC (GF-250 column, Bio-Rad, Hercules CA) run at 1 mL/minute in 0.2 M acetate buffer, pH 5.0, with a UV detector set at 496 nm, essentially one detected peak elutes near the retention time of the hLL1 antibody. The trace (*see* Figure 1; UV detector at 496 nm, set to detect DOX) shows doxorubicin-LL1 conjugate as essentially a single peak at retention time of around nine minutes, without aggregated proteinaceous species or free DOX (retention time around 14 minutes). This indicates that very little free or no drug is present in the product. Samples of DOX-hLL1 conjugate are aliquoted into single fractions, typically of 0.1-1.0 mg, and frozen for future use, or alternatively they are lyophilized. They are defrosted or reconstituted, as needed, for further testing.

Example 5: Coupling of doxorubicin to hLL1 and formulation of the dox-hLL1 conjugate

[0081] a) Reaction of doxorubicin with SMCC hydrazide

[0082] Mix 90 mg of doxorubicin (1.56×10^{-4} mol) and 60.23 mg of SMCC hydrazide in 13 mL of 1:2 methanol:ethanol (anhydrous), and add 10.4 µL of trifluoroacetic acid. The mixture is allowed to stir for 4 h, in the dark, at room temperature. The reaction solution is then filtered through a 0.22 micron syringe filter into a 100 mL round-bottomed flask. Seventy-five µL of diisopropylethylamine is added and the solvent evaporated on a rotary evaporator at 300° C. The residue is triturated with 4 x 40 mL acetonitrile followed by 1 x 40 mL diethyl ether and dried to a powder on the rotary evaporator under high vacuum. The powder was redissolved in 5 mL anhydrous methanol, re-evaporated to dryness as above, and then stored at -200° C until needed.

[0083] b) Reduction of hLL1-IgG with dithiothreitol

[0084] In a 20 mL round bottomed flask are mixed 8.4 mL of hLL1-IgG (10.3 mg/mL, 5.78×10^{-7} mol), 160 µL of 0.1 M sodium phosphate buffer pH 7.5, 500 µL of 0.2 M

EDTA, pH 7.0, and 290 μL of deionized water. The mixture is deoxygenated by cycling solution six times between vacuum and an argon atmosphere. A freshly prepared solution of 40 mM dithiothreitol (DTT) in water (0.015g in 2.4 mL water, 2.3 x 10^{-5} mol; 40-fold molar excess to IgG) is deoxygenated by bubbling argon through it for 10 minutes, and 640 μL of this aqueous DTT solution is added to the deoxygenated hLL1 antibody solution. The resulting mixture is incubated at 37° C for 1 hour. The reduced antibody is purified by diafiltration (one 30K filter, under argon, at 4° C), against deoxygenated 10 mM PBS/100 mM L-histidine, pH 7.4, buffer. The buffer is added continuously until total filtrate volume is 300 mL. The volume of the reduced hLL1 solution (hLL1-SH) is reduced to 10 mL.

[0085] c) Conjugation of doxorubicin-SMCC to hLL1-SH and purification of conjugate

[0086] The activated doxorubicin (1.9 mL, 2.09 x 10^{-5} mol, 36-fold excess to IgG) is taken up in dimethylsulfoxide (DMSO) solution and then slowly added to the hLL1-SH antibody solution (40 mL) under argon at room temperature. The final concentration of DMSO is 5%. The reaction is allowed to proceed with gentle stirring for 40 minutes at 4° C. The reaction mixture is loaded onto a BioBeadTM (Bio-Rad, Richmond CA) column (1.5 cm diameter x 34 cm high, equilibrated with 10 mM PBS/100 mM L-histidine, pH 7.4, buffer), and run through at 2 mL/min. The product conjugate is concentrated in an Amicon filtration unit and filtered through a 0.22 micron syringe filter prior to formulation for lyophilization.

[0087] d) Conjugate formulation and lyophilization

[0088] To 40 mL of the above hLL1-dox solution are added 8 mL of 0.5M mannitol solution in water, and 0.48 mL of 1% polysorbate 20, resulting in final concentrations of 1.64 mg/mL hLL1-dox, 82.5 mM mannitol, and 0.01% polysorbate-20. Samples are lyophilized in 1 mg and 10 mg dox-hLL1 quantities (3 and 10 mL vials, respectively), frozen on dry ice, and lyophilized under vacuum over 48 h. Vials are stoppered under vacuum, and stored sealed at –20° C, in the dark, for future use.

Example 6: Preparation of morpholino-DOX and cyanomorpholino-DOX conjugates of antibodies

[0089] Morpholino-DOX and cyanomorpholino-DOX are prepared by reductive alkylation of doxorubicin with 2,2'-oxy-bis[acetaldehyde], using the procedure of Acton, *et al.* (*J. Med. Chem.* *27*:638-645 (1984)).

[0090] These DOX analogs were coupled with M_2C_2H in the same manner as described above for the DOX and 2-PDOX analogs. Cyanomorpholino-DOX was coupled with 10 % molar excess of the hydrazide in anhydrous methanol (instead of DMSO) overnight at the room temperature. Solvent removal, followed by flash chromatography furnished the hydrazone. Electrospray mass spectral analysis: M+H m/e 872, M+Na 894; M-H 870. In a similar fashion, morpholino-DOX was derivatized to its hydrazone using SMCC-hydrazide using 1.5 equivalent of the reagent in anhydrous methanol for 4 h, and the product was purified by flash chromatography. Electrospray mass spectrum: M+H m/e 847, M-H m/e 845, M+Cl m/e 881.

[0091] Interchain disulfide bonds of antibodies were reduced to free thiols as described above in Examples 2-4, to generate disulfide-reduced mAbs, and conjugates were prepared using the same methods as described in section c) of each of Examples 2, 3, and 4. The following mAb conjugates of morpholino-DOX and cyanomorpholino-DOX were prepared:

morpholino-DOX-antibody conjugates:

mRS7 conjugate: drug-to-mAb substitution ratio: 6.4:1.

mMN-14 conjugate: drug-to-mAb substitution ratio: 8.9:1.

cyanomorpholino-DOX-antibody conjugates:

mRS7 conjugate: drug-to-mAb substitution ratio: 5.3:1.

mMN-14 conjugate: drug-to-mAb substitution ratio: 7.0:1.

Example 7: In Vitro Efficacy of Anthracycline-Antibody Conjugates

[0092] Raji B-lymphoma cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD), and were grown in RPMI 1640 medium containing 12.5% fetal bovine serum (Hyclone, Logan, UT), supplemented with glutamine, pyruvate, penicillin and streptomycin (Life Technologies, Grand Island, NY). Briefly, 3.75 x 10^5 cells were incubated for 2 days with the indicated concentration of drug-mAb conjugate in 1.5 mL of tissue culture medium in wells of 24-well plates. The cells were then transferred to T25 flasks containing 20 ml of medium, and incubated for up to 21 days, or until the cells had multiplied 16-fold. Viable cell counts using Trypan blue were performed at day 0, day 2, and then every 3-5 days. From the growth rate of untreated cells, the doubling time was calculated, and the Fraction Surviving was calculated from the time required for treated cells to multiply 16-fold, assuming that the doubling time was not affected by treatment. A single

remaining viable cell could be readily detected. At a concentration of drug-mAb conjugate of 1 μg/mL the DOX-LL1 conjugate shows a three-orders of magnitude difference in the fraction of surviving cells, in comparison to the DOX-MN-14 conjugate. *See* Figure 2.

Example 8: Treatment of Tumor-Bearing Animals with Anthracycline-Antibody Conjugates

[0093] a) Treatment in a solid tumor xenograft model. Groups of athymic nude mice were injected subcutaneously with DU145 human prostate cancer cells. After approximately two weeks, when palpable prostate tumor xenografts had grown in the animals, half were treated with a single dose of the drug-antibody conjugate 2-PDOX-RS7, and half were left untreated (controls). Figure 3, shows the growth of the tumor xenografts in untreated mice versus the growth of xenografts in mice treated with 2-PDOX-RS7. It shows a therapeutic effect for animals treated with the drug-antibody conjugate, in terms of delayed growth of the xenografts.

[0094] b) Treatment of systemic cancer in an animal model. NCr-SCID mice, in groups of ten animals, were each given an intravenous injection of a suspension of 2.5×10^6 cells of the human Burkitt's B-cell lymphoma cell line, Raji, by tail-vein injection. Five days later, animals were left untreated or treated with single doses of either 350 μg DOX-LL1 or 150 μg 2-PDOX-LL1. Figure 4 shows the result of the experiment. Untreated animals become paralyzed and die at around 23 days post-injection of the Raji cells, from systemic cancer. Animals treated with DOX- and 2-PDOX-conjugates of the LL1 antibody survived over an extended period corresponding to around a four-fold increase in life expectancy for the 2-PDOX-LL1-treated animals, and an even greater increased life expectancy for the DOX-LL1-treated animals.

[0095] c) Treatment of systemic cancer in an animal model. NCr-SCID mice, in groups of ten animals, were each given an intravenous injection of a suspension of 2.5×10^6 cells of the human Burkitt's B-cell lymphoma cell line, Raji, by tail-vein injection. Five days later, animals were left untreated or treated with single doses of either 150 μg 2-PDOX-LL1 or 150 μg of 2-PDOX-MN-14 (non-specific control antibody conjugate). Figure 5 shows the result of the experiment. Untreated animals become paralyzed and die at around 23 days post-injection of the Raji cells, from systemic cancer, as do animals treated with the 2-PDOX-MN-14 conjugate. Animals treated with the 2-PDOX-LL1 antibody conjugate survive over an extended period.

[0096] From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usage and conditions without undue experimentation. All patents, patent applications and publications cited herein are incorporated by reference in their entirety.

Cited References

U.S. Patent Documents:

Arcamone *et al.*, U.S. Patent No. 4,125,607

Greenfield *et al.*, U.S. Patent No. 5,122,368

Janaky *et al.*, U.S. Patent No. 6,214,969

Kaneko *et al.*, U.S. Patent No. 5,349,066

Kaneko *et al.*, U.S. Patent No. 5,137,877

McKenzie *et al.*, U.S. Patent No. 5,798,097

King *et al.*, U.S. Patent No. 6,307,026

King *et al.*, U.S. Patent No. 5,824,805

King *et al.*, U.S. Patent No. 5,162,512

Moreland *et al.*, U.S. Patent No. 5,241,078

Schally *et al.*, U.S. Patent No. 6,184,374

Schally *et al.*, U.S. Patent No. 5,843,903

Willner *et al.*, U.S. Patent No. 5,708,146

Willner *et al.*, U.S. Patent No. 5,622,929

Willner *et al.*, U.S. Patent No. 5,606,017

Other References:

Anthracycline Antibiotics: New Analogs, Methods of Delivery, and Mechanisms of Action. *ACS Symposium Series 574*, W. Priebe, editor, Publ. American Chemical Society, 1995.

Acton *et al., J. Med. Chem.*, 27:638-645, 1984.

Arencibia *et al., Anticancer Drugs,* 12:71-78, 2001.

Benali *et al., Proc. Natl. Acad. Sci. U.S.A.,* 97:9180-9185, 2000.

Chastzistamou *et al., Clin. Cancer Res.,* 6:4158-4165, 2000.

Denmeade *et al., Cancer Res.,* 58:2537-40, 1998.

Dubowchik *et al., Bioconjug. Chem.*, 13:855-869,2002.

Halmos *et al., Cancer Lett.,* 136:129-136, 1999.

Hansen *et al., Biochem J.,* 320:393-300, 1996.

Kahan *et al., Breast Cancer Res. Treat.,* 59:255-262, 2000.

Kahan *et al., Int. J. Cancer,* 82:592-598, 1999.

Kahan *et al., Cancer* 85:2608-2615, 1999.

Kiaris *et al., Eur. J. Cancer,* 37:620-628, 2001.

Kiaris *et al., Br. J. Cancer,* 81:966-971, 1999.

Koppan *et al., Cancer Res.,* 58:4132-4137, 1998.

Krebs *et al., Cancer Res.,* 60:4194-4199, 2000.

Michel *et al., Clin. Cancer Res.,* 8:2632-2639, 2002.

Miyazaki *et al., Am. J. Obstet. Gynecol.* 180:1095-103, 1999.

Miyazaki *et al., J. Natl. Cancer Inst.,* 89:1803-1809, 1997.

Mosure *et al., Cancer Chemother. Pharmacol.,* 40:251-258, 1997.

Nagy *et al., Proc. Natl. Acad. Sci. U.S.A.,* 97:829-834, 2000.

Nagy *et al., Proc. Natl. Acad. Sci. U.S.A.,* 95:1794-1799, 1998.

Nagy *et al., Proc. Natl. Acad. Sci. U S A.,* 94:652-656, 1997.

Nagy *et al., Proc. Natl. Acad. Sci. U.S.A.,* 93:7269-7273, 1996.

Nagy *et al., Proc. Natl. Acad. Sci, U.S.A.,* 93:2464-2469, 1996.

Ong *et al. Immunology,* 98:296-302, 1999.

Pawlyk-Byczkowska *et al., Cancer Res.,* 49:4568-4577, 1989.

Plonowski *et al., Int. J. Cancer,* 88:652-657, 2000.

Plonowski *et al., Cancer Res.,* 60:2996-3001, 2000.

Plonowski *et al., Cancer Res.,* 59:1947-1953, 1999.

Roche *et al., Proc. Natl. Acad. Sci. U.S.A.,* 90:8581-8585, 1993.

Schally *et al., Clin. Cancer Res.,* 7:2854-2861, 2001.

Schally *et al., Prostate,* 45:158-166, 2000.

Schally *et al., Eur. J. Endocrinol.,* 141:1-14, 1999.

Shih *et al. Cancer Immunol. Immunother.,* 49:208-216, 2000.

Suzawa *et al., J. Contr. Release,* 79:229-242, 2002.

Westphalen *et al., Int. J. Oncol.* 17:1063-1069, 2000.

Wraight *et al., J. Biol. Chem.,* 265:5787-5792, 1990.